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Roles of Thapsigargin-Sensitive Ca^{2+} Stores in the Survival of Developing Cultured Neurons

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Abstract: The roles of the intracellular calcium pool involved in regulating the Ca^{2+} profile and the neuronal survival rate during development were studied by using thapsigargin (TG), a specific inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase in cultured cerebellar granule neurons. Measuring the neuronal $[Ca^{2+}]_i$ directly in the culture medium, we found a bell-shaped curve for $[Ca^{2+}]_i$ versus cultured days in cerebellar granule neurons maintained in medium containing serum and 25 m*M* K^+. The progressive increase in $[Ca^{2+}]_i$ of the immature granule neurons (1–4 days in vitro) was abolished by TG, which resulted in massive neuronal apoptosis. When the $[K^+]$ was lowered from 25 to 5 m*M,* neither the progressively increasing $[Ca^{2+}]_i$ nor the survival of immature granule neurons was significantly changed over 24-h incubation. Similarly, TG caused a dramatic decrease in the $[Ca^{2+}]_i$ and survival rate of these immature neurons when switched to 5 m*M* K^+ medium. Following maturation, the granule neurons became less sensitive to TG for both $[Ca^{2+}]_i$ and neuronal survival. However, TG can protect mature granule neurons from the detrimental effect of switching to a 5 m*M* K^+ serum-free medium by decreasing $[Ca^{2+}]_i$ to an even lower level than in the respective TG-free group. Based on these findings, we propose that during the immature stage, TG-sensitive ER Ca^{2+}-ATPase plays a pivotal role in the progressive increase of $[Ca^{2+}]_i$, which is essential for the growth and maturation of cultured granule neurons. **Key Words:** Calcium—Endoplasmic reticulum—Thapsigargin—Neuronal survival.
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Cerebellar granule neurons are among the most abundant neuronal phenotypes in the mammalian CNS (Yan et al., 1995). During cerebellar ontogeny, only those neurons that acquire appropriate neurotrophic factors survive (Levick et al., 1995). Besides neurotrophic factors, the essential electric activities for neuronal survival during development have been emphasized in many studies (Meriney et al., 1987; Maderdrut et al., 1988; Johnson et al., 1997). In concert with this contention, Balázs et al. (1988*a*,*b*) suggested that the survival-promoting effect of high K^+ on cultured cerebellar granule neurons was due to membrane depolarization in vitro simulating the effect of the earliest afferent inputs received by the granule cells in vivo. This in turn might be mediated through the stimulation of excitatory amino acid receptors, in particular, the *N*-methyl-D-aspartate-preferring subtype gating ion channels that are also permeable to Ca^{2+}.

In culture, cerebellar granule neurons isolated from 7- or 8-day postnatal rats can survive and acquire the morphological, biochemical, and electrophysiological characteristics of mature neurons in medium containing serum and 25 m*M* K^+ (HK) (Scott and Fisher, 1970; Collins et al., 1991; Franklin and Johnson, 1992; Levick et al., 1995; Skaper et al., 1998). Following maturation, these cultured granule neurons reliably undergo apoptotic death when switched to serum-free medium with 5 m*M* K^+ (NK) (Levick et al., 1995; Chang and Wang, 1997; D'Mello et al., 1998; Skaper et al., 1998). Therefore, this paradigm was used by many investigators to study the mechanisms underlying apoptosis in the CNS.

Many reports have also suggested that the sustained rise in neuronal $[Ca^{2+}]_i$ is the critical biochemical event responsible for the survival-promoting effect of HK conditions (Gallo et al., 1987; Collins et al., 1991; Koike and Tanaka, 1991; Franklin and Johnson, 1992), and most of them focus on the contribution of Ca^{2+} channels. In comparison with the literature concerning voltage-gated Ca^{2+} channels, much less is known about the contribution of internal Ca^{2+} pools, such as the endoplasmic reticulum (ER), on the $[Ca^{2+}]_i$ of cultured neurons.

In this study, an ER Ca^{2+}-ATPase blocker, thapsigargin (TG) (Thastrup et al., 1990; Mason et al., 1991), was adopted to investigate this theme in cerebellar granule neurons 24 h after plating. Measuring the neuronal $[Ca^{2+}]_i$ directly in the culture medium, we can demon-

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Abbreviations used: ACSF, artificial cortical spinal fluid; BME, basal medium Eagle; DIV, days in vitro; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; HK, high extracellular K^+ (25 m*M*); NGF, nerve growth factor; NK, normal extracellular K^+ (5 m*M*); TG, thapsigargin.

strate that the TG-sensitive Ca^{2+}-ATPase is indispensable for the continuously increasing $[Ca^{2+}]_i$ during development. Without the function of this Ca^{2+}-ATPase, the increasing $[Ca^{2+}]_i$ values of immature neurons were almost completely diminished and subsequently resulted in neuronal apoptosis. Although TG became much less toxic to mature neurons in HK medium, it exhibited neuroprotective effects when the mature neurons were switched to NK serum-free medium. Under this condition, TG still caused an even lowered level of $[Ca^{2+}]_i$. These results suggest that TG-sensitive Ca^{2+}-ATPase, which is responsible for the progressive increase of $[Ca^{2+}]_i$, is indispensable in regulating $[Ca^{2+}]_i$ and survival of these developing cultured neurons.

MATERIALS AND METHODS

Preparation of cultured cerebellar granule neurons

Rat cerebellar granule neurons were prepared from 7-day-old Wistar rats, as previously described (Levi et al., 1984). In brief, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase and then seeded onto poly-L-lysine (Sigma)-coated 22-mm-diameter coverslips. Cells were seeded at a density of 2×10^6 cells/coverslip in basal medium Eagle (BME; Gibco) supplemented with 10% fetal calf serum (Gibco), 25 m*M* KCl, penicillin G (100 IU/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cytosine arabinoside (10 μM) was added to the culture medium 18–24 h after plating to arrest the growth of nonneuronal cells.

Treatment of cultures

For immature granule neurons, treatments were initiated 24 h after plating. Neurons were treated with TG [RBI; dissolved in dimethyl sulfoxide (DMSO) and diluted in artificial cortical spinal fluid (ACSF) to a final 0.025% DMSO concentration] in original medium or immediately after they were transferred into NK serum-containing medium. The same amounts of DMSO and ACSF were added for control experiments. For experiments with mature neurons, treatments were performed at 7–8 days in vitro (DIV). The procedures were the same as the ones for the immature cells, except that to avoid the toxic effects of fresh serum, culture medium was substituted with NK serum-free medium when the medium was switched from HK to NK (Levick et al., 1995; Chang and Wang, 1997; D'Mello et al., 1998; Skaper et al., 1998).

Measurement of $[Ca^{2+}]_i$ of granule neurons

Measurement of $[Ca^{2+}]_i$ was carried out as previously described by using microspectrofluorimetry and the Ca^{2+}-sensitive indicator fura-2 (Sigma) (Grynkiewicz et al., 1985; Yan et al., 1995; Lin et al., 1998). In brief, granule neurons grown on poly-L-lysine-coated 22-mm-diameter coverglass were loaded for 30 min at 37 ± 0.5°C with fura-2-AM (5 μM) applied in culture medium. The medium containing fura-2-AM was then gently removed and replaced with the corresponding culture medium saved earlier. Coverslips were placed in a thermostatted (37 ± 0.5°C) stage on a Zeiss Axiovert 135-TV inverted microscope. Excitation of fura-2 was at 340 and 380 nm with emitted light monitored at 510 nm. Cell-derived fluorescent images were visualized using a 40× 1.3-NA oil immersion objective (Fluar) and captured by an OlymPix 50 2500 cooled CCD camera (Life Science Resources, Cambridge, U.K.). The digitized images were then calculated for ratio images using the Merlin image-processing package (Life Science Resources). Calcium concentration was calculated according to the formula as follows (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = K_D [(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$, where $K_D = 285$ n*M* (Groden et al., 1991; Yan et al., 1995) and S_{f2} and S_{b2} are the emitted fluorescence intensities (510 nm) excited by 380 nm at R_{min} and R_{max}, respectively. The values of R_{max} and R_{min} were obtained from average values of the calibration procedure using ionomycin (10 μM) and EGTA (5 m*M*).

For studying the acute effect of TG on $[Ca^{2+}]_i$, treatments were performed directly on the stage of microscope. The effect of prolonged exposure was measured after incubation with TG for the specific time periods indicated in the legends.

Neuronal survival

Viable granule neurons were quantified after loading with fura-2-AM (5 μM), which is kept in only by living cells. After measurement of $[Ca^{2+}]_i$, fluorescent images of viable neurons were taken randomly and the numbers of living cells per whole screen image were counted. Values are generally expressed as percentages of untreated controls in each experiment.

Morphological methods

Cerebellar granule neurons were observed by using both Nomarski optics and fluorescent staining with Hoechst 33258 to examine morphological evidence of apoptosis (Dipasquale and Youle, 1992) and nuclear condensation/aggregation (Oberhammer et al., 1992; Yanagihara and Tsumuraya, 1992), respectively. For staining with Hoechst 33258, the neurons were washed with phosphate-buffered saline (pH 7.4) and fixed for 10 min in 4% formaldehyde in phosphate-buffered saline at 4°C, washed with distilled water, and dried at room temperature (Oberhammer et al., 1992). Neurons were then stained with Hoechst 33258 (5 μg/ml) for 5 min, washed, and dried. Photomicrographs of Hoechst 33258 and Nomarski optics were obtained, respectively, from a cooled CCD camera (OlymPix 50 2500) described above and a Contax camera (167 MT) adapted to the Zeiss Axiovert 135-TV microscope.

Statistical analysis

All data are presented as means ± SE. Statistical significance ($p < 0.05$) was assessed by ANOVA followed by Dunnett's *t* tests.

RESULTS

$[Ca^{2+}]_i$ profile of cerebellar granule neurons developing in HK serum-containing medium

As shown in Fig. 1, cerebellar granule neurons grown in the HK culture medium for 1 day had a $[Ca^{2+}]_i$ of 176 ± 9 n*M* (n = 87). The $[Ca^{2+}]_i$ progressively increased to 758 ± 15 n*M* (n = 148) at 3–4 DIV and then gradually decreased to 258 ± 5 n*M* (n = 151) at 7 DIV. Thus, there appears to be a bell-shaped curve between neuronal $[Ca^{2+}]_i$ versus DIV (Fig. 1). The neurons mature with the dynamic changes in $[Ca^{2+}]_i$ level during 7-day culture.

No effect of lowering K^+ from 25 to 5 m*M* on $[Ca^{2+}]_i$ accumulation in immature neurons

Upon switching of the immature granule neurons (1 DIV) from HK to NK culture medium for another 24 h, the neuronal survival and morphology were similar in both conditions as shown in Figs. 2B and 3, respectively.

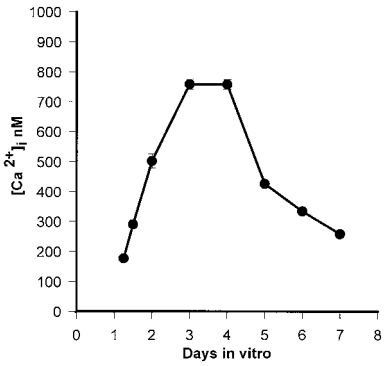

FIG. 1. Intracellular calcium profile of cerebellar granule neurons developing in HK serum-containing medium. A representative experiment with a bell-shaped curve of $[Ca^{2+}]_i$ versus days after culture is shown. Granule neurons were obtained from 7-day-old rats and cultured in BME supplemented with 10% fetal calf serum and 25 m*M* KCl. Mean ± SE values are plotted, n = 87–151. Similar results were obtained from the other four individual experiments.

The effect of switching from HK to NK serum-containing medium on the $[Ca^{2+}]_i$ of immature neurons was explored in this study. As shown in Fig. 2A, switching to NK medium did not lower the $[Ca^{2+}]_i$; the neuronal $[Ca^{2+}]_i$ stayed high for another 24 h. Besides, these immature neurons switched to NK serum-containing medium tended to have slightly higher $[Ca^{2+}]_i$ levels than control groups at the time intervals of 6, 16, and 24 h. However, the acute change of intracellular calcium could not be detected within 10 min after switching to NK serum-containing medium (362 ± 21 n*M* before and 376 ± 26 n*M* after, n = 76, $p = 0.68$, respectively) (Fig. 4A). As shown in Figs. 2 and 3, immature granule neurons could survive in the absence of HK stimulation, and this was perhaps due to the similar leveling of $[Ca^{2+}]_i$ during this 24-h period.

Contribution of ER to $[Ca^{2+}]_i$ of immature neurons developing in vitro

In addition to HK-evoked transmembrane Ca^{2+} influx through the Ca^{2+} channel, we explored in this study the participation of the ER in regulating the $[Ca^{2+}]_i$ of cultured granule neurons. Treatment with the ER Ca^{2+}-ATPase blocker TG (500 n*M*) abolished the expected $[Ca^{2+}]_i$ accumulation in 6- to 24-h incubation, leading to a massive neuronal death (Fig. 2); the concentration-dependent effect of TG on neuronal survival is shown in Fig. 2C. After 6-h treatment with TG, $[Ca^{2+}]_i$ significantly decreased as compared with the respective control either in HK or in NK medium (Fig. 2A), but the survival rate was not significantly changed (Fig. 2B). Upon further incubation for 16–24 h, $[Ca^{2+}]_i$ remained at lower levels in TG groups, but the control group gradually increased in a time-dependent manner. The survival rate significantly decreased after 16-h TG treatment and was almost abolished by the 24-h treatment. This massive death of immature granule neurons induced by TG is due to apoptosis, as revealed by Nomarski optics and staining with Hoechst 33258 (Fig. 3). However, the immediate effect of TG on the $[Ca^{2+}]_i$ within 10-min incubation in the immature granule neurons (1 DIV) was negligibly detectable whether they were measured in HK medium (346 ± 42 and 361 ± 40 n*M* before and 10 min after TG treatment, respectively; n = 58, $p = 0.79$) or NK serum-containing medium (373 ± 28 and 352 ± 31 before and 10 min after TG treatment, respectively; n = 76, $p = 0.63$) (Fig. 4B).

Decrease of dependence of $[Ca^{2+}]_i$ and neuronal survival on ER Ca^{2+}-ATPase following maturation

Following maturation, blocking the ER Ca^{2+}-ATPase of mature granule neurons (7 DIV) by TG for short periods of time (10 min) also failed to alter the $[Ca^{2+}]_i$ homeostasis (372 ± 28 and 352 ± 31 n*M*

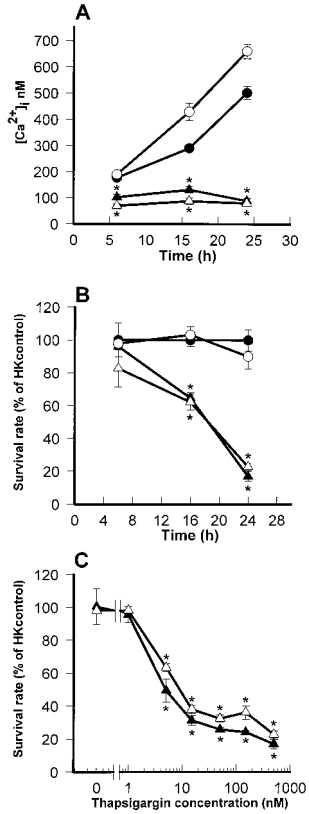

FIG. 2. Changes in $[Ca^{2+}]_i$ and survival of immature granule neurons after prolonged treatment with TG. Immature granule neurons (1 DIV) were treated with 500 n*M* TG in HK medium or switched to NK serum-containing medium with TG. **A** and **B:** Time course of TG-induced changes in $[Ca^{2+}]_i$ (n = 58–255) and survival (performed in triplicate) of immature granule neurons, respectively. (●), untreated control in HK medium; (▲), TG treated in HK medium; (○), switched to NK serum-containing medium; (△), switched to NK serum-containing medium with TG. **C:** Dose–response analysis. Cytotoxic effect induced by various concentrations of TG was quantified 24 h after treatment. (▲), TG treated in HK medium; (△), switched to NK serum-containing medium with TG. The value of the untreated control group is set as 100%. Data are plotted as means ± SE. Similar results were obtained from the other three individual experiments. *$p < 0.05$ as compared with the respective TG-free control group.

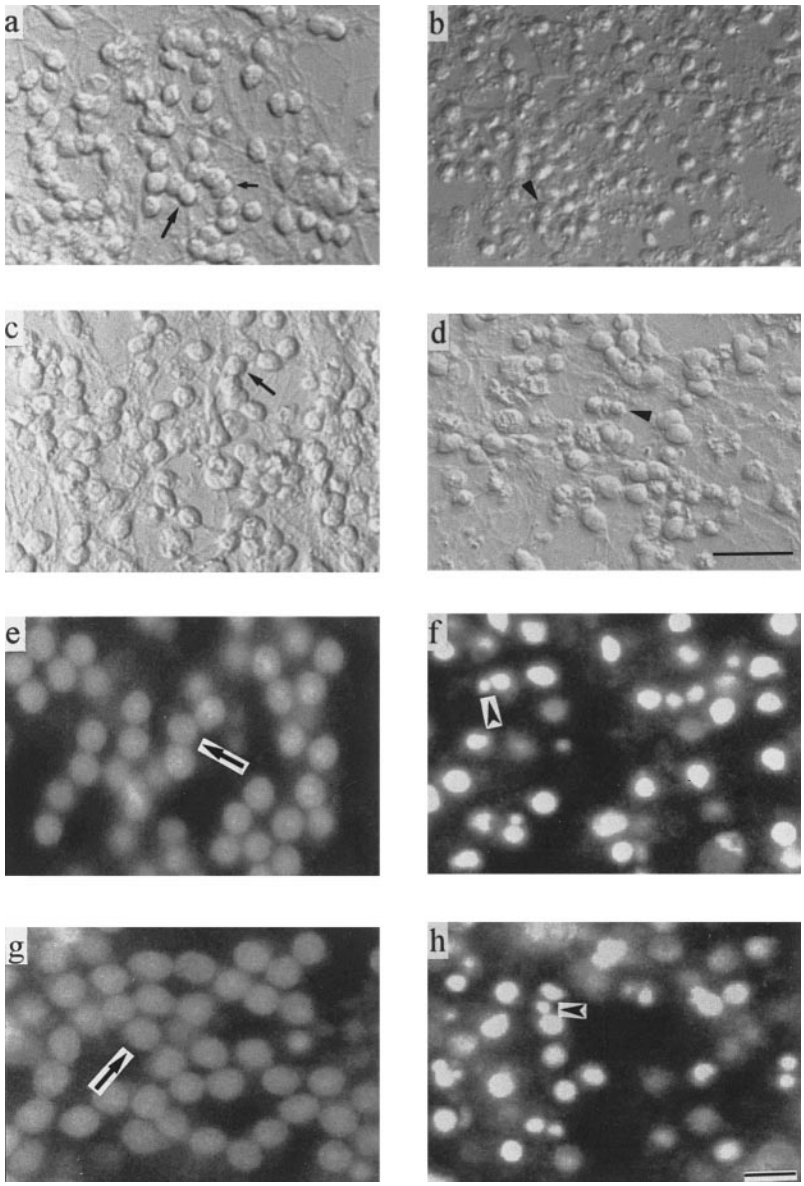

FIG. 3. Representative morphological features of apoptotic cell death of immature granule neurons induced by 500 n*M* TG. The cellular morphology was observed by Nomarski optics (**a–d**) and after staining with the fluorescent dye Hoechst 33258 (**e–h**). Immature granule neurons (1 DIV) were maintained in HK medium (a and e), treated with TG in HK medium (b and f), or switched to NK serum-containing medium (c and g) or NK serum-containing medium with TG (d and h). Morphological features were examined 24 h after treatment. Arrows indicate neurons with normal morphology, whereas arrowheads indicate apoptotic neurons. Bar = 25 μm (a–d) and 10 μm (e–h).

before and after treatment with TG in HK medium, respectively; $p = 0.82$, n = 100). Even with a prolonged (24-h) treatment with TG in these mature granule neurons in HK medium, the percentage of $[Ca^{2+}]_i$ change (2.3%) was nonsignificant in contrast to that occurring in immature neurons (82.5%), as shown in Figs. 2A and 5A.

The only slightly reduced neuronal survival rate determined 24 h after challenge with 500 n*M* TG in HK medium as compared with that in immature neurons ($89.7 \pm 2.3\%$ survival in mature group and $16.8 \pm 3.0\%$ in immature group) (Figs. 2B and 5B) may be due to the diminution in change of $[Ca^{2+}]_i$. As shown in Fig. 6a, b, e, and f, the morphology of the cells in the TG-treated group appeared to be the same as that in the control group.

Neuroprotective effect and even lower $[Ca^{2+}]_i$ caused by TG in mature granule neurons after switching to NK serum-free medium

The fully differentiated granule neurons (7 DIV) died by apoptosis in a time-dependent manner (Fig. 5B) after switching from HK to NK serum-free medium (Fig. 6c and g) that could be reversed by TG treatment (Fig. 6d and h). We measured the $[Ca^{2+}]_i$ of the mature neurons in NK serum-free medium after treatment with TG to investigate the possible mechanism behind this prevention. The $[Ca^{2+}]_i$ values of mature granule neurons were dramatically reduced from 392 ± 14 to 97 ± 3 n*M* when we switched them from HK to NK serum-free medium (Fig. 7A), which presumably accounted for the subsequent massive neuronal apoptosis (Figs. 5 and 6c and g). As shown in Fig. 7B, TG cannot replace HK to keep the

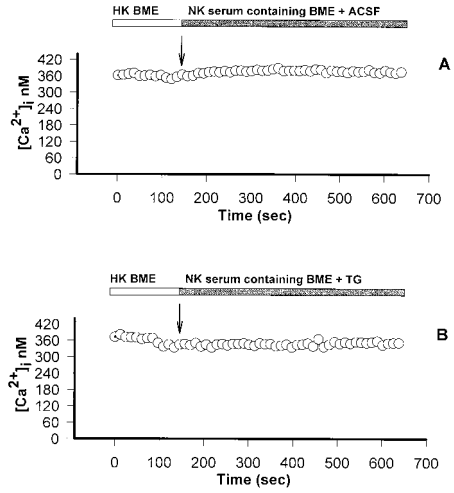

FIG. 4. Representative effect of TG on the $[Ca^{2+}]_i$ of immature granule neurons (1 DIV) after being switched to NK serum-containing medium (indicated by the arrow) during a 10-min period. Mean $[Ca^{2+}]_i$ values of immature granule neurons were calculated and plotted after treatment with vehicle ACSF as a control (n = 76) (**A**) or treatment with 500 n*M* TG (n = 76) (**B**) on the temperature-controlled stage of the microscope. Similar results were obtained from the other three individual experiments.

$[Ca^{2+}]_i$ of mature neurons elevated (320 ± 11 and 84 ± 3 n*M* before and after treatment of TG immediately after being switched to NK serum-free medium). Moreover, TG caused an even lower $[Ca^{2+}]_i$ than in the TG-free group after 7-h treatment in NK serum-free medium (Fig. 5A) and perhaps thus prevented the subsequent neuronal death caused by NK serum-free medium (Fig. 5B and C). The differences in neuronal survival between the TG-treated and TG-free group after 24-h treatment were 92.1 ± 1.8 and 24.2 ± 1.8%, respectively (Fig. 5B). Meanwhile, the measured $[Ca^{2+}]_i$ of TG-treated neurons stayed at a relatively lower level than that without TG (28.3 ± 1.1 and 42.6 ± 4.5 n*M*, respectively; Fig. 5B). This result indicated that TG depleted the Ca^{2+} required for HK deprivation-induced death of mature granule neurons rather than replaced HK to maintain the elevated $[Ca^{2+}]_i$.

DISCUSSION

It is well established that neurotrophic factors are required for cultured neurons (Koike and Tanaka, 1991; Eichler et al., 1992; Park et al., 1997). High $[K^+]$ instead of neurotrophic factors has been found to be sufficient in maintaining the survival of cerebellar granule neurons (Balázs et al., 1988*a*,*b*; Levick et al., 1995; Skaper et al., 1998) and other neuronal types in culture (Collins et al., 1991; Franklin and Johnson, 1992). It has been found that the $[Ca^{2+}]_i$ of cultured neurons growing with the support of nerve growth factor (NGF) progressively increased with days in culture and reached a plateau later on (Koike and Tanaka, 1991; Eichler et al., 1992). Although it is well known that the survival-promoting effect of HK is associated with a sustained rise of $[Ca^{2+}]_i$ (Gallo et al., 1987; Collins et al., 1991; Koike and Tanaka, 1991; Franklin and Johnson, 1992), little is known about the $[Ca^{2+}]_i$ profile of CNS neurons developing in HK medium. In this report, we have demonstrated for the first time a bell-shaped curve for $[Ca^{2+}]_i$ versus culture days of cerebellar granule neurons maintained in HK medium. As shown in Fig. 1, the neuronal $[Ca^{2+}]_i$ progressively increased to a peak at 3–4 DIV and then went down to a lower level (6–7 DIV). The increasing phase appears to be ultimately correlated with the differentiation of immature granule neurons and supports them being exempt from apoptosis. Meanwhile, the decline phase may be due to the resting of $[Ca^{2+}]_i$ homeostasis caused by maturation. In addition to the Ca^{2+} influx induced by HK, we were prompted to explore the role of internal Ca^{2+} stores like ER on $[Ca^{2+}]_i$ and

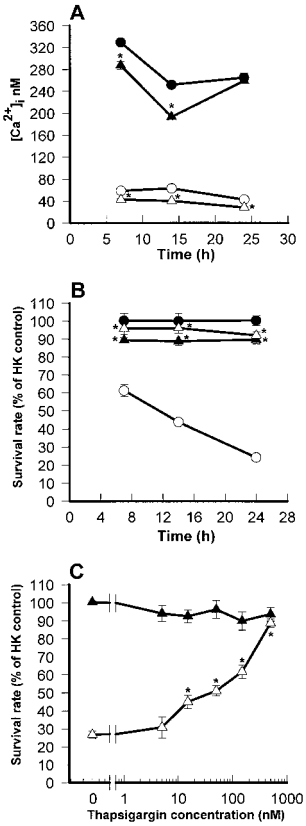

FIG. 5. Changes in $[Ca^{2+}]_i$ and survival of mature (7 DIV) granule neurons after prolonged treatment with TG. **A** and **B:** Time course of TG-induced changes in $[Ca^{2+}]_i$ (n = 201–426) and survival (performed in triplicate) in mature granule neurons. (●), untreated control; (▲), treated with 500 n*M* TG in HK medium; (○), switched to NK serum-free medium; (△), switched to NK serum-free medium with TG. **C:** Mature granule neurons were treated with various concentrations of TG in HK medium (▲) or switched to NK serum-free medium with various concentrations of TG (△). Neuronal survival was quantified 24 h after treatment. Data are plotted as means ± SE. The value of the untreated control group is set as 100%. Similar results were obtained from the other three individual experiments. $^*p < 0.05$ as compared with respective TG-free control group.

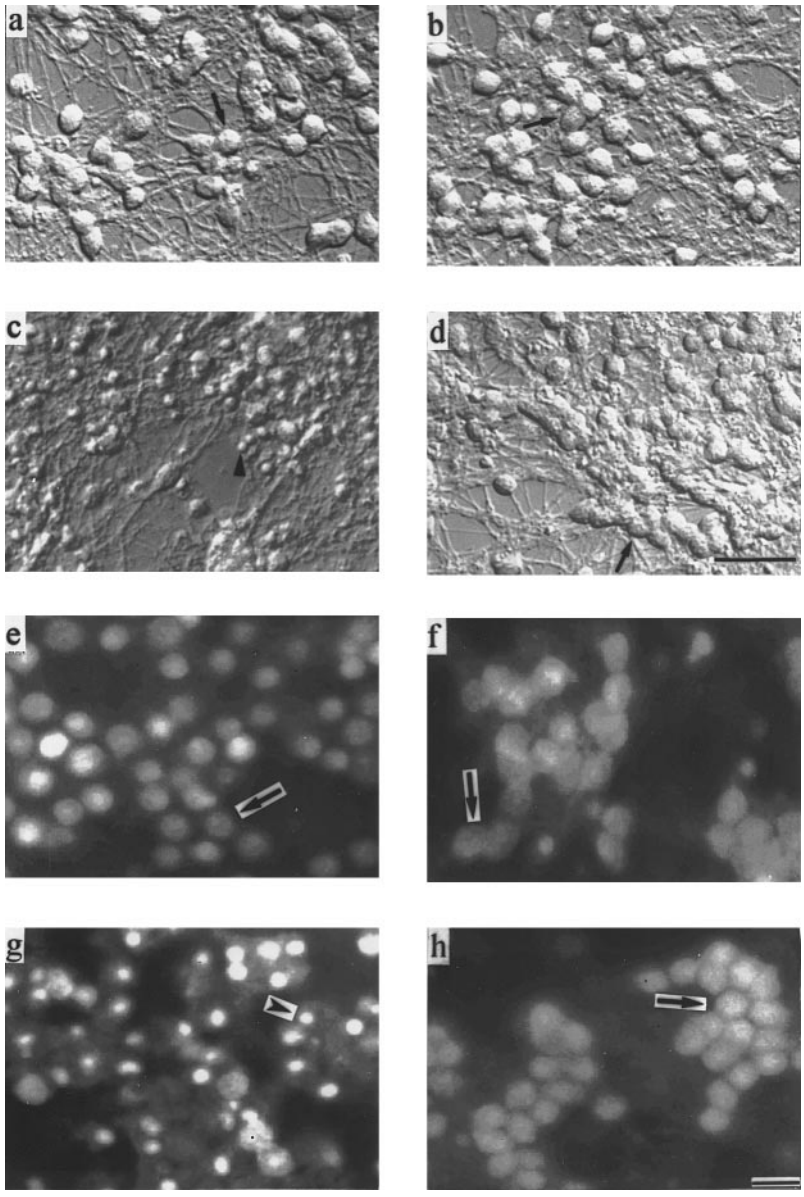

FIG. 6. Representative morphological features of mature granule neurons (7 DIV) induced by 500 n*M* TG. The cellular morphology was examined by Nomarski optics (**a–d**) and after staining with the fluorescent dye Hoechst 33258 (**e–h**). Mature granule neurons were maintained in HK medium (a and e), treated with TG in HK medium (b and f), or switched to NK serum-free medium (c and g) or NK serum-free medium with TG (d and h). Morphological features were examined 24 h after treatment. Arrows indicate neurons with normal morphology, whereas arrowheads indicate apoptotic neurons. Bar = 25 μm (a–d) and 10 μm (e–h).

survival. The specific ER Ca^{2+}-ATPase inhibitor TG (Thastrup et al., 1990; Lytton et al., 1991) was used to elucidate this specific aim. We found that TG treatment abolished the elevated $[Ca^{2+}]_i$ accumulation, suggesting that the rising phase of $[Ca^{2+}]_i$ was profoundly dependent on the function of TG-sensitive Ca^{2+} stores rather than the elevated extracellular $[K^+]$. Once the neuronal $[Ca^{2+}]_i$ surpassed the peak level, the influence of TG on neuronal $[Ca^{2+}]_i$ and survival was dramatically changed and also became susceptible to the toxic effects of fresh serum, as documented by other studies (Aloisi et al., 1985; Schramm et al., 1990). The decreasing phase of this curve is different from the plateau phase of other types of neurons induced by NGF (Koike and Tanaka, 1991; Eichler et al., 1992). These differences may be due to different neuronal types, methodology of measurement, or natural properties evoked by NGF and HK. Ciardo and Meldolesi (1991) also demonstrated that the transient $[Ca^{2+}]_i$ response to the challenge of HK stimulation progressively increased in developing cerebellar granule neurons and declined after 6–7 DIV. Even though these two results were measured under different conditions, the declining phase in both may be due to the natural course of maturation. At present, the mechanism underlying this decreasing phase is still unclear.

Our findings of similar $[Ca^{2+}]_i$ levels of immature neurons cultured in either HK or NK medium provide part of the answer for the HK-independent neuronal survival during early stages of development. As shown in Fig. 2A, the progressive increase of early neuronal $[Ca^{2+}]_i$ did not change after a switch to NK medium. It thus seems that in developing neurons, the sustained rise

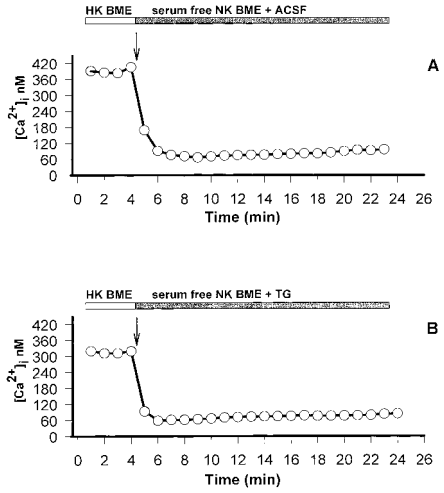

FIG. 7. Representative effect of TG on $[Ca^{2+}]_i$ of mature granule neurons (7 DIV) after being switched to NK serum-free medium (indicated by the arrow) during a 20-min period. Mean $[Ca^{2+}]_i$ values of mature granule neurons were calculated and plotted after treatment with vehicle ACSF as a control (n = 27) (**A**) or treatment with 500 n*M* TG (n = 31) (**B**) on the temperature-controlled stage of the microscope. Similar results were obtained from the other three individual experiments.

of $[Ca^{2+}]_i$ depends on factors other than Ca^{2+} influx activated by HK stimulation. These may include the effects of an internal calcium buffering system and serum activation. In comparison with the significant advances made in our understanding of how HK-activated Ca^{2+} channels act in the survival of cultured neurons (Gallo et al., 1987; Koike et al., 1989; Collins et al., 1991; Franklin and Johnson, 1992; Becherer et al., 1997), much less is known about the contribution of internal Ca^{2+} pools like ER. Thus, TG was employed to determine the influence of ER Ca^{2+} stores on the developing neuronal $[Ca^{2+}]_i$ and survival. TG had been shown to increase $[Ca^{2+}]_i$ in many cell types (Thastrup et al., 1990; Llopis et al., 1991; Lytton et al., 1991; Mason et al., 1991; Sagara and Inesi, 1991). In thymocytes, apoptosis induced by TG required influx of extracellular Ca^{2+} and could be prevented by chelators of extracellular Ca^{2+} (Jiang et al., 1994). However, unlike what was expected by other reports (Levick et al., 1995), we found that TG killed those immature neurons grown in both HK and NK medium by abolishing the progressive increase in $[Ca^{2+}]_i$ rather than by causing a $[Ca^{2+}]_i$ overload. This would give a reasonable explanation for the results reported by Levick et al. (1995) that KCl, Ca^{2+} channel blockers, and MK-801 could not prevent the TG-induced apoptosis in early granule neurons grown in NK medium. TG was also reported to induce death in embryonic chick nodose neurons (Larmet et al., 1992) and cause widespread neuronal injury in perinatal rat brain (Silverstein and Nelson, 1992). It is also reasonable for us to deduce that TG may additionally inhibit the $[Ca^{2+}]_i$ accumulation of those neuronal populations rather than elevate it to a lethal concentration. These findings provide information on the newly found functions of TG-sensitive Ca^{2+} stores in regulating $[Ca^{2+}]_i$ and survival of developing neurons. This is in agreement with the reports (Ghosh et al., 1991; Short et al., 1993) showing that TG can lower the resting $[Ca^{2+}]_i$ of the DDT_1MF-2 smooth muscle cell line and arrest cell proliferation. However, TG can induce a transient $[Ca^{2+}]_i$ rise in DDT_1MF-2 cells (Ghosh et al., 1991) but not in our cultured granule neurons (a positive control in astrocytes).

After maturation, we found that the granule neurons became resistant to TG regarding both neuronal survival and $[Ca^{2+}]_i$ levels in HK medium. As a result of this discovery, we deduced that following maturation, the $[Ca^{2+}]_i$ regulatory system may change to a state less dependent on TG-sensitive Ca^{2+}-ATPase and shift to rely on the HK-evoked transmembrane Ca^{2+} influx.

Although mature neurons become less sensitive to TG, we have demonstrated that TG exerted a neuroprotective effect in the detrimental switching to an NK serum-free medium. Regarding this positive effect of TG on the survival of mature neurons in NK serum-free medium, one would expect TG to cause rising $[Ca^{2+}]_i$ values in mature granule neurons (Levick et al., 1995). However, measurement of $[Ca^{2+}]_i$ in these mature neurons during TG treatment revealed that TG further lowered the $[Ca^{2+}]_i$ rather than maintaining the elevated level. Therefore, this explains the finding that neither MK-801 nor L-type Ca^{2+} channel blockers could alter the positive effect of TG on the survival of mature neurons in NK serum-free medium (Levick et al., 1995). It is considered that after depletion of $[Ca^{2+}]_i$ by TG, the apoptotic signal transmitted by Ca^{2+} from the ER may be blocked and thus prevent the subsequent neuronal death. This working hypothesis is corroborated by the report that the interleukin-1β-converting enzyme-like protease could be activated when mature granule neurons were switched to NK medium (Armstrong et al., 1997; D'Mello et al., 1998). The Ca^{2+} from ER may be involved in the activation of this interleukin-1β-converting enzyme-like protease and therefore cause the neuronal apoptosis. This hypothesis is partially in agreement with a report from Malcolm et al. (1996) that stabilizing internal Ca^{2+} storage by TMB-8 could prevent the Ca^{2+}-dependent component of glutamate neurotoxicity. The critical role of internal Ca^{2+} storage on neuronal death was also noted in recent studies describing the protective effects of TMB-8 and dantrolene on excitotoxicity (Frandsen and Schousboe, 1991; Lei et al., 1992; Mody and MacDonald, 1995).

The "Ca^{2+} setpoint" hypothesis postulates that depending on its level, Ca^{2+} can either support neuronal survival or cause death (Koike et al., 1989; Koike and Tanaka, 1991; Johnson and Deckwerth, 1993; Levick et al., 1995). The profound effects of TG on $[Ca^{2+}]_i$ levels in immature neurons, as shown in our results, indicate that neuronal death at this stage is due to lack of sufficient $[Ca^{2+}]_i$ rather than overloading. The bell-shaped curve of $[Ca^{2+}]_i$ seems to show that immature neurons can tolerate considerable amounts of $[Ca^{2+}]_i$ during de-

velopment. Following maturation, cellular properties such as the Ca^{2+} buffering system and the biochemical machinery responding to TG may change and cause the different responses to TG, as shown in our results. These effects of TG on neuronal $[Ca^{2+}]_i$ and survival in different stages of development clearly elucidate the differential function of ER Ca^{2+}-ATPase.

The influence of ER Ca^{2+}-ATPase on $[Ca^{2+}]_i$ may be different in different cell types, for example, granule neurons, thymocytes, and DDT_1MF-2 cells. We could not demonstrate a TG-induced $[Ca^{2+}]_i$ rise, but Ghosh et al. (1991) observed a transient $[Ca^{2+}]_i$ rise in DDT_1MF-2 cells. The so-called capacitative calcium influx activated by depletion of TG-sensitive Ca^{2+} stores was not demonstrated in the granule neurons. This is consistent with the recent report by Kiedrowski and Costa (1995); they tentatively explained this finding as follows: (1) There is insufficient Ca^{2+} pumping by the ER Ca^{2+}-ATPase, (2) the Ca^{2+} leak from ER is slow, (3) the TG-sensitive Ca^{2+} stores are not abundant in cerebellar granule neurons, and (4) there are additional TG-insensitive mechanisms mediating Ca^{2+} uptake into the ER. However, we definitely showed the blocking effect of TG on $[Ca^{2+}]_i$ accumulation accompanied by massive neuronal death after prolonged 24-h incubation with the immature neurons, suggesting that normal functions of ER Ca^{2+}-ATPase participated in maintaining the appropriate $[Ca^{2+}]_i$. Based on these findings, we propose that TG-sensitive Ca^{2+}-ATPase is essential for Ca^{2+} accumulation in the ER of immature neurons, which transmit the signal for growth and development. Prolonged treatment with TG leads to loss of the Ca^{2+}-sequestering effect, resulting in a dramatic lowering of $[Ca^{2+}]_i$. Barish (1998) has pointed out that Ca^{2+}-sequestering organelles such as the ER provide both an internal Ca^{2+} regulation and distribution system and a scaffold for synthesis, targeting, and insertion of the functional membrane proteins. TG apparently caused the massive neuronal apoptosis mediated by abolishing this crucial signaling for the vital synthetic machinery.

In conclusion, we have demonstrated for the first time in this study that dynamic changes (a bell-shaped profile) of $[Ca^{2+}]_i$ play a crucial role in the development and maturation of cerebellar granule neurons cultured in vitro. Our results suggest that TG-sensitive Ca^{2+}-ATPase is critical in this functional $[Ca^{2+}]_i$ profile and neuronal survival. Failure to regulate $[Ca^{2+}]_i$ can lead to neuronal apoptosis. A variety of acute and chronic neuronal diseases such as ischemic stroke injury, Parkinson's disease, and Alzheimer's disease have shown signs of neuronal apoptosis mediated by an increase in $[Ca^{2+}]_i$ (Harada and Sugimoto, 1997; Skaper et al., 1998). Therefore, this newly found function of TG-sensitive Ca^{2+} stores implies an alternative direction in considering the etiology and management of these diseases.

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